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Blood Protein Markers of Neocortical Amyloid- β Burden: A Candidate Study Using SOMAscan Technology

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Abstract.

Background: Four previously reported studies have tested for association of blood proteins with neocortical amyloid- β burden (NAB). If shown to be robust, these proteins could have utility as a blood test for enrichment in clinical trials of Alzheimer's disease (AD) therapeutics.

Objective: This study aimed to investigate whether previously identified blood proteins also show evidence for association with NAB in serum samples from the Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing (AIBL). The study considers candidate proteins seen in cohorts other than AIBL and candidates previously discovered in the AIBL cohort.

Methods: Our study used the SOMAscan platform for protein quantification in blood serum. Linear and logistic regressions were used to model continuous NAB and dichotomized NAB respectively using single proteins as a predictor. Multiple protein models were built using stepwise regression techniques and support vectors machines. Age and *APOE* ϵ 4 carriage were used as covariates for all analysis.

Results: Of the 41 proteins previously reported, 15 AIBL candidates and 20 non-AIBL candidates were available for testing. Of these candidates, pancreatic polypeptide (PPY) and IgM showed a significant association with NAB. Notably, IgM was found to associate with continuous NAB across cognitively normal control subjects.

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Conclusions: We have further demonstrated the association of PPY and IgM with NAB, despite technical differences between studies. There are several reasons for a lack of significance for the other candidates including platform differences and the use of serum rather than plasma samples. To investigate the possibility of technical differences causing lack of replication, further studies are required.

Keywords: Alzheimer's disease, amyloid plaques, blood, positron emission tomography scan, proteomics

INTRODUCTION

The pathology of AD is characterized by three features in the brain: the aggregation of amyloid- β (A β) into plaques, the presence of hyperphosphorylated tau in the form of tangles, and the occurrence of neuron loss leading to brain atrophy [1, 2]. There is debate as to how these processes interact to cause symptomatic AD. A popular theory is the amyloid cascade hypothesis: that A β deposition is central to disease development [3]. Studies show that A β plaques begin to develop up to 20 years prior to clinical diagnosis with their growth reaching a plateau as clinical symptoms arise [4, 5]. The presence of AD pathology can be investigated through characteristics that are informative of AD diagnosis; endophenotypes. Measurements of analytes in cerebrospinal fluid (CSF) and molecular imaging by positron emission tomography (PET) scans are examples of endophenotypes that are biomarkers for AD. The disadvantages of the procedures involved in attaining these measurements include their invasive or expensive nature and that they require specialized administration.

Existing treatments for AD provide short-term symptomatic relief, in a subset of patients, and trials of potential disease modifying treatments are failing [6]. Two suggestions have been made to address this shortcoming: the use of A β as a companion diagnostic in tertiary prevention trials (those investigating disease modifying or symptomatic treatments) and in secondary prevention trials (investigating treatments to prevent the disease from occurring in the first place). Firstly, we consider the use of a companion diagnostic in a normal clinical trial. To date, trials of A β targeting drugs have recruited patients with a clinical AD or mild cognitive impairment (MCI) diagnosis and unknown brain A β burden [7–10]. As A β PET scans became more readily available, toward the end of such trials, A β burden was measured and it was discovered that some of these AD patients had low A β burden (the target pathology). For example, in a trial of Bapineuzumab, a humanized anti-A β monoclonal antibody, approximately 14% of subjects had low A β burden [9]. Consequently, it has been suggested that

A β PET or CSF measures are used as a companion diagnostic, with elevated brain A β becoming an eligibility requirement for A β -lowering trials. A panel of blood biomarkers could enable cost-effective enrichment and identification of trial participants with A β pathology for subsequent confirmatory A β CSF tests or PET scans.

A second use of a blood-based biomarker would be in recruitment for secondary prevention trials. It is hypothesized that the brains of people recruited with a diagnosis of AD or MCI are often too damaged for medication to have an effect. Treatments could have a higher chance of success in subjects with no clinical symptoms, under the assumption that it is easier to delay, rather than reverse, the development of AD pathology. Aisen et al. expand on this idea stating that there has been no medical advancements in the last decade in terms of treating AD [11]. They suggest basing recruitment of patients into clinical trials on the presence of biomarker defined pre-symptomatic AD; three such trials had begun by the end of 2014. As above, using a blood biomarker as an enrichment filter for PET scans and CSF markers could increase the efficiency of clinical trial recruitment by reducing screen failures. A simple blood test has the greatest potential impact in streamlining secondary prevention studies as screen failure rates due to low A β are expected to be higher ($\sim 78.1\%$ versus $\sim 12\%$) in asymptomatic individuals than in subjects with AD (Ossenkoppele et al. and Jansen et al., unpublished results).

Consequently, there is high demand for a blood-based biomarker of AD pathology [12]. It has been shown that measures of A β in the brain are highly negatively correlated with those in the CSF [13]. However, overall, studies of blood A β do not show similar correlations [14]. It is therefore necessary to search for other analytes within the blood that may be associated with A β in the brain. Recent studies provide some evidence that proteins in blood show such a link to other AD endophenotypes and to A β burden in the brain [15–19]. However, there is a need to investigate these results further in independent datasets of a larger size, using different technologies and different biological samples. We begin to address this here.

This study aimed to further test findings in the literature of proteins in blood plasma associating with brain A β burden [15–17, 19]. We used a radioactive marker of A β , [^{11}C]-Pittsburgh compound B (PiB), combined with PET scanning to quantify the amount of brain A β burden and the SOMAscan proteomics platform [20] for quantification of protein in blood samples.

With over 1,000 protein analytes, the SOMAscan proteomics platform has been used in high-throughput biomarker discovery studies. For example, in AD research it has been used by Sattlecker et al. and Zhao et al. to discover potential plasma biomarkers of AD diagnosis, MRI measures, and/or rate of cognitive decline [18, 21]. In this study we focused our approach on candidate-based analysis, selecting only proteins previously found to associate with NAB. This was done to ensure that there was sufficient statistical power to detect weaker signal that could have been missed had the entire SOMAscan panel been used. This analysis was repeated within just the cognitively normal control subjects to evaluate whether this test would have utility to enrich for A β -positivity in secondary prevention trials.

MATERIALS AND METHODS

Cohort

AIBL is a prospective, longitudinal study of subjects aged over 60 years. The dual center study recruits subjects with an AD diagnosis as well as both control subjects and those with MCI with the aim of identifying factors that lead to subsequent AD development. Details of study design and enrolment are given elsewhere [17]. The present study comprised a subset of 198 subjects from AIBL, enriched for controls.

Proteomics

Blood draws (80 ml) were taken after overnight fasting and serum was collected in Sarstedt s-monovette tubes. The samples were centrifuged at 1,800 g for 15 min at 20°C and immediately frozen at –80°C. They were stored (long-term) in liquid nitrogen [22]. Protein levels in the blood serum were analyzed using the SOMAscan platform (SomaLogic Inc, Boulder, CA). The methods used in this assay are outlined in detail by Kraemer et al. but, in brief, proteins were measured using Slow Off-rate Modified Aptamer (SOMAmer)-based capture arrays using a sample of less than 10 μL per run [23]. SOMAmers are nucleotides that have been chemically modified to

address two issues: hard to capture proteins and non-specific binding. Firstly, the nucleotides are given protein-like properties resulting in high affinity for hard to capture proteins. Secondly, aptamers with slow dissociation rates are used allowing disruption of non-specific binding. These nucleotides are used to transform a protein signal to a nucleotide signal that can be quantified using relative fluorescence on microarrays. For full quantification details of this assay, see Gold et al. [20]. This study used SOMAscan Version 2 which captured information on 1,001 proteins and protein complexes.

Quality control was performed at the sample and SOMAmer level, and involved the use of control SOMAmers on the microarray and calibration samples. Hybridization controls measured sample-by-sample variation in hybridization while the median signal over all SOMAmers measured technical variability. Scale factors of these two metrics were used to normalize across all samples with acceptance criteria of 0.4 to 2.5 based on historic trends. SOMAmer by SOMAmer calibration occurred through the repeated measurement of calibration samples. Historic values were used to generate a calibration scale factor the acceptance criterion for which was: 95% of SOMAmers must have a calibration scale factor within 0.4 of the median.

Imaging

The measures of NAB used in this study were collected by PiB PET scans. The PiB imaging methodology is detailed elsewhere [24]. Each scan was spatially normalized by Rowe et al. to a customized PiB-PET template in the Montreal Neurological Institute reference space using Statistical Parametric Mapping 8 (SPM8; Wellcome Trust Centre for Neuroimaging, London, UK). Standardized uptake value ratios (SUVRs) were then created by computing the ratio of PiB retention in the whole brain to that in the grey matter [25].

Identification of candidate blood protein markers of brain A β burden

We identified candidate proteins through a search of the literature for studies investigating the relationship between blood proteomics and A β burden in the brain. Any proteins identified as significant in the study conclusions were selected and matched to the AIBL SOMAscan data using the UniProt ID.

Statistical analysis

All statistical analysis was performed in R (version 3.1.1) [26].

Differences in proteomic signal between platforms and sample types

As the candidates were selected based on their association with NAB in plasma, we aimed to confirm that serum is an appropriate surrogate by comparing proteomic signal from the SOMAscan platform between blood plasma and serum samples using age as an outcome. We also studied differences between the SOMAscan platform and Myriad's Rules Based 415 Medicine Multi-analytes Profile (RBM MAP), one of the discovery proteomics platforms. To do so, we used proteomic data generated using the RBM MAP platform from the original publication of a blood biomarker in the AIBL study; methods described by Burnham et al. [17].

Menni et al. have studied associations of age with protein levels from plasma samples using the TwinsUK cohort and SOMAscan version 3 [27]. Running random intercept linear regression, adjusting for family relatedness, identified proteins associated with chronological age. We performed a similar analysis using standard linear regression on our serum samples from the AIBL cohort. Within each cohort (TwinsUK and AIBL) the proteins were ranked by *p*-value from the regressions and the R package *OrderedList* was used to compare the rank of proteins present on both versions of the platform [28].

To assess the concordance of proteomic signal between SOMAscan and RBM MAP, we implemented an identical method. *OrderedList* was used to compare lists of proteins ranked according to their associations with age and gender.

Analysis overview

An overview of the analysis is given in Fig. 1. Proteins discovered in studies other than AIBL [15, 16] were analyzed separately to proteins discovered in AIBL using different biological samples (serum instead of plasma) [17, 19]. This split was implemented as the association of a non-AIBL candidate with NAB in AIBL would provide a fully independent replication of those findings, whereas the association of an AIBL candidate with NAB would only show that the marker could also be measured using SOMAmers on serum samples. Each analysis tested the protein levels

against the continuous endpoint of SUVR as well as a dichotomized high/low NAB endpoint. A cut off of 1.3 was used to distinguish between high and low NAB [17]. Use of a continuous endpoint gives increased power in statistical analysis in comparison to a categorical measure [29]. Additionally, each analysis studied all samples and control samples alone.

Subjects were randomly split into a training (66%) and test set (34%) using the *createDataPartition* function in the R package *caret* [30]. The split ensured that a range of values of NAB were present in each set for the continuous analysis by splitting the subjects into groups based on NAB percentiles and sampling within these groups. For the dichotomized analysis, the split ensured that the proportion of high and low NAB was similar between the two sets by sampling within the high and low groups.

Data pre-processing

Data pre-processing was performed separately for each analysis. In both cases, all protein data was subject to a natural logarithm transformation so each protein followed an approximate normal distribution. Per sample, per protein outliers were then identified as values lying outside of 6 standard deviations (SD) of the mean protein value and were set to missing. Any sample with more than 20% missing data was removed. Missing data in the train and test sets was imputed separately using 10 nearest neighbors.

In some cases, there were multiple SOMAscan probes available for one UniProt ID. In this instance, a principal components analysis (PCA) was performed across these probes and only the first principal component (PC1) was included in the analysis. This was performed on all samples and on control samples alone, and separately for train and test sets. Additionally, the collinearity of such probe sets was investigated using Pearson's correlation coefficients.

Single protein analysis

Models were built using the training set. The continuous and dichotomized NAB endpoints were regressed against age, APOE ϵ 4 load and each protein in turn using linear and logistic regression, respectively. Age and APOE ϵ 4 load, defined as the number of ϵ 4 alleles in a subjects genotype, were included as they were statistically significant in this population (see Table 2).

In all cases, Benjamini Hochberg corrected *p*-values from the full model fit (*q*-values) were tested against a significance level of 0.1, to account for false discov-

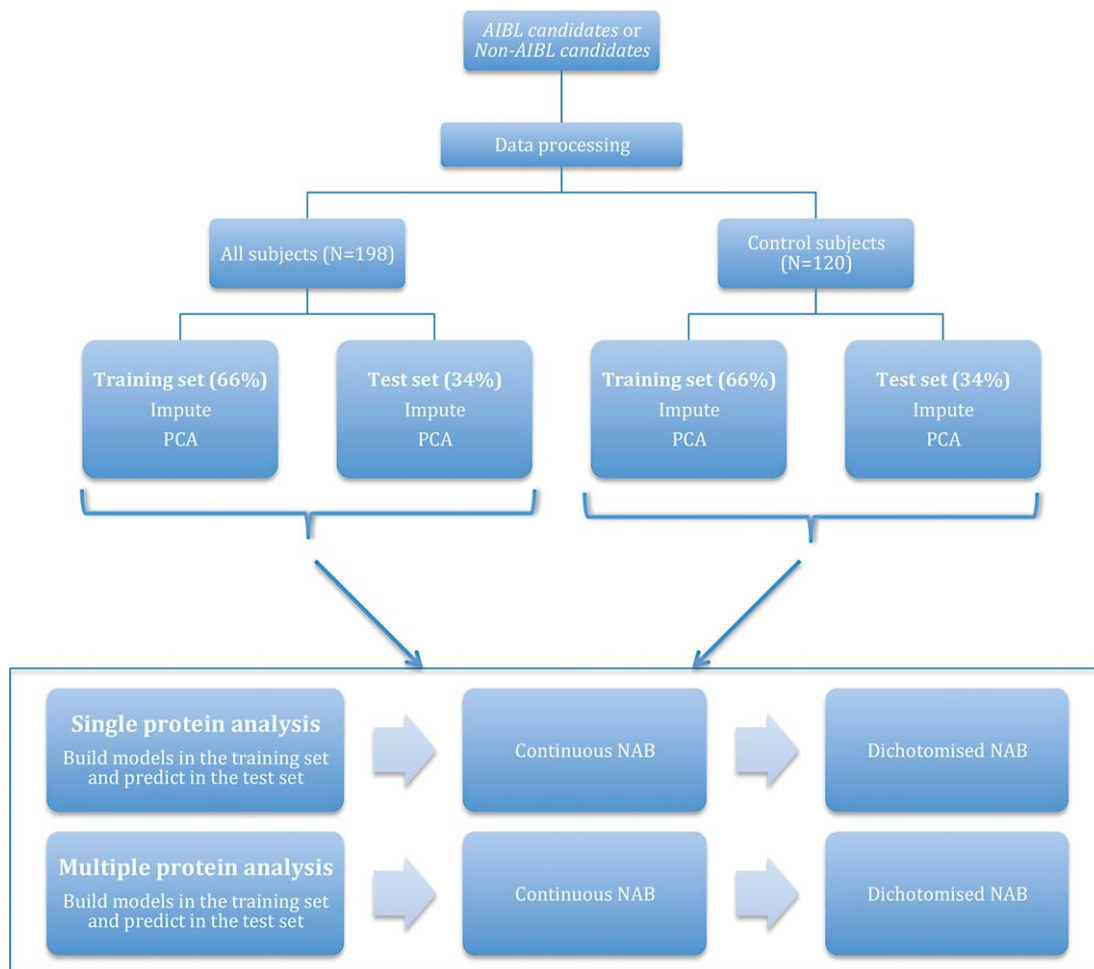


Fig. 1. Overview of data analysis.

ery. Although the hypothesis tests performed were not independent, the Benjamini Hochberg correction gave an acceptable approximation of the false discovery rate [31].

Model fit was assessed by exploring the residuals of each model to ensure normality, constant variance, zero expectation, and independence. For logistic regression models, deviance residuals were used.

Each model was used to predict NAB in the test set and model statistics were calculated. These were R^2 and root mean squared error (RMSE) for linear regression and accuracy, sensitivity and specificity for logistic regression. For each model, statistic permutation testing was run with 1,000 permutations of the NAB values, to create a non-parametric, empirical p -value. In each permutation, the relationship between NAB endpoint, age, and APOE $\epsilon 4$ load was maintained. Benjamini Hochberg corrected p -values (q -values) were tested against a significance level of 0.1.

Multiple protein analysis

The primary multiple protein analysis was parametric. Models were built using the training set. As in the single protein analysis, continuous and dichotomized NAB endpoints were regressed against age, APOE $\epsilon 4$ load, and all proteins together (15 non-AIBL candidates and 20 AIBL candidates) using linear and logistic regression respectively. The models were then simplified using stepwise regression based on Akaike's Information Criteria (AIC). Simplified linear regression models were examined for variable importance using the Lindemann, Merenda, and Gold (LMG) measure [32]. This metric represents the R^2 of an individual regressor by averaging over all orders of regressors. Each model was used to predict on the test set and create model statistics with associated empirical p -values, as detailed previously. Model fit was assessed in the same manner as for the single protein analysis.

As a secondary multiple protein analysis we investigated the possibility of non-linear associations and interactions by performing a non-parametric multiple protein analysis using support vector machines (SVMs) in the R package kernlab [33]. We used an SVM with radial kernel to build models in the training set. The train function from the R package caret was used to estimate model parameters: The kernel parameter (sigma) was directly estimated and regularization parameter (C) was tuned using 25 bootstraps. RMSE was used as the optimization metric for continuous NAB while kappa was used for the dichotomized endpoint. Each model was used to predict on the test set.

RESULTS

Cohort demographics

For this study, we used SOMAscan data generated on serum samples from the AIBL cohort. Table 2 summarizes the demographics of this population. As expected, characteristic features of AD such as Mini-Mental State Examination score and Clinical Dementia Rating were significantly associated with continuous NAB and significantly different between high and low NAB groups. Age and APOE ϵ 4 load (defined as the number of APOE ϵ 4 alleles in a subject's genotype) were also significant and hence accounted for in all analyses.

Overall, demographics in the control population mirrored those across all samples and hence the same terms were covaried for in the control only analyses (see Supplementary Table 1). As expected, measures of APOE ϵ 4 were not significant in this sub-population.

Differences in proteomic signal between platforms and sample types

We began by comparing proteomic signal associated with age in plasma samples from the TwinsUK cohort and serum samples from AIBL. 975 SOMAscan probes matched between the Twins UK (Version 3, 1,129 SOMAmers) and AIBL (Version 2, 1,001 SOMAmers) datasets mapped using SOMAscan ID. Comparison of the protein lists ranked by association with age gave a significance of similarity p -value of <0.001 . The analyses were not adjusted for any covariates.

We also made a comparison between the SOMAscan platform and RBM MAP platform using samples from the AIBL cohort. The RBM MAP data contained 151 proteins, 119 of which could be mapped to a UniProt ID. All 1,001 proteins on the SOMAscan panel were mapped to a UniProt ID. There were 88 proteins

that overlapped between the two datasets, mapped by UniProt ID. Comparison of the protein lists ranked by association with age gave a significance of similarity p -value of 0.162. An identical analysis with proteins ranked by association with gender gave a significance of similarity p -value of 0.201.

Identification of candidate blood protein markers of brain A β burden

We identified four previous studies investigating a proteomic signal in the blood associated with A β burden in the brain. Previously reported studies used blood plasma whereas this study uses blood serum samples. Thambisetty et al. discovered six proteins associated with brain A β burden in a group of non-demented, older individuals [15]. Kiddle et al. used data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) in a discovery analysis to find 16 proteins associated with brain A β burden [16]. Significant proteins from these two papers that were also present in the AIBL SOMAscan data [15] are termed non-AIBL candidates and are given in Table 1.

Additionally, two discovery analyses have already been performed on AIBL subjects using different protein measurement technologies; RBM MAP [17] and mass spectrometry [19]. Respectively, these studies highlighted 8 and 17 proteins (a prioritized set) as being significantly associated with NAB. The proteins that were also present in the AIBL SOMAscan data [20] are termed AIBL candidates and are given in Table 1. See Supplementary Figure 1 for an overview of protein numbers.

In some cases, there were multiple SOMAscan aptamers for one UniProt ID: C3 (P01024) had six matches in the AIBL SOMAscan dataset, APOE (P02649) had three, and Fibrinogen ([P02671, P02675, P02679]) had two. All are accounted for in this analysis using PCA as detailed previously. Eleven of the 41 proteins were not present on the SOMAscan panel. A β PP was included as an additional protein of interest as A β _{1–42} is not present on the SOMAscan panel.

Data pre-processing

There was no observed pattern in the missing data nor in the demographics of the subjects with data set to missing so it was assumed the data were missing at random. No subjects were removed during data pre-processing.

The protein data was generated in one batch and the PET scans were performed using one model of

Table 1
Candidate proteins

Protein	Gene	UniProt ID	Ashton et al. [19]	Burnham et al. [25]	Kiddle et al. [24]	Thambisetty et al. [23]	Total	Comments
Complement C3	C3	P01024	X		X	X	3	5 probes present.
Apolipoprotein E	APOE	P02649			X	X	2	3 probes present.
Fibrinogen (α , β , γ)	FG	P02671, P02675, P02679	X		X		2	3 probes present. Separate signal from α and γ chains [19]
Haptoglobin	HP	P00738	X			X	2	
Pancreatic Polypeptide	PPY	P01298		X	X		2	
A β 1-42				X			1	Not present in AIBL SOMAscan.
Albumin	ALB	P02768				X	1	
Alpha 1 Antitrypsin	SERPINA1	P01009			X		1	
Alpha 2 Macroglobin	A2M	P01023	X				1	
Apolipoprotein A	LPA	P08519	X				1	Not present in AIBL SOMAscan.
Apolipoprotein A1	APOA1	P02647	X				1	
Apolipoprotein A4	APOA4	P06727	X				1	Not present in AIBL SOMAscan.
Apolipoprotein L1	APOL1	O14791	X				1	Not present in AIBL SOMAscan.
AXL Receptor Tyrosine Kinase	AXL	P30530	X		X		1	Not present in AIBL SOMAscan.
C-Peptide	INS	P01308			X		1	
C4b Binding Protein Alpha Chain	C4BPA	P0C0L4	X				1	
Chemokine Ligand 13	CXCL13	O43927		X			1	
Clusterin	CLU	P10909	X				1	
Complement Factor B	CFB	P00751	X				1	
Complement Factor H	CFH	P08603	X				1	
Complement Factor H Related Protein 1	CFHR1	Q03591	X				1	Not present in AIBL SOMAscan.
Cortisol	(N/A)	(N/A)			X		1	
Free Thyroxine	(N/A)	(N/A)		X			1	Not present in AIBL SOMAscan.
Gelsolin	GSN	P06396	X				1	Not present in AIBL SOMAscan.
Hemopexin	HPX	P02790	X				1	
Histidine-rich Glycoprotein	HRG	P04196	X				1	
Ig Gamma 1 Chain C Region	IGHG1	P01857				X	1	
Immunoglobulin E	(N/A)	(N/A)			X		1	Not present in AIBL SOMAscan.
Immunoglobulin M 1	IGHM	P01871		X			1	
Interleukin 13	IL13	P35225			X		1	
Interleukin 17	IL17	Q16552		X			1	
Interleukin 3	IL3	P08700			X		1	
Leptin	LEP	P41159			X		1	
Macrophage Inflammatory Protein (1 α)	CCL3	P10147		X			1	
Matrix Metalloproteinase 9 Total	MMP9	P14780			X		1	
Plasminogen	PLG	P00747				X	1	
Serotransferrin	TF	P02787	X				1	
Serum Amyloid p-Component	APCS	P02743			X		1	
Vascular Cell Adhesion Protein	VCAM1	P19320		X			1	Not present in AIBL SOMAscan.
Vitronectin	VTN	P04004			X		1	
Von Willebrand Factor	VWF	P04275			X		1	
Amyloid- β Protein Precursor	A β PP	P05067					0	Included as an alternative to A β 1-42

Table 2
Population demographics

	Total <i>n</i> = 198	Overall <i>p</i> -value	High NAB <i>n</i> = 107	Low NAB <i>n</i> = 91	High/Low <i>p</i> -value
Gender (% female)	50	0.96	49	52	0.78
<i>APOE</i> status (% of <i>APOE</i> ϵ 4 positive)	51	<0.001	61	50	0.004
<i>APOE</i> ϵ 4 load (% with loads 0; 1; 2)	49; 43; 8.1	<0.001	39; 48; 13	60; 37; 2.2	0.002
Median age [IQR] (years)	72 [14]	<0.001	76 [11]	68 [12]	<0.001
Median MMSE score [IQR]	28 [3.0]	<0.001	28 [4.0]	29 [2.0]	<0.001
Global CDR status (%>0)	44	<0.001	64	21	<0.001
Clinical diagnosis (% with diagnosis AD; MCI; Control)	14; 26; 61	0.28	25; 35; 40	0; 15; 85	<0.001

Individuals were positive for *APOE* ϵ 4 if at least one *APOE* ϵ 4 allele was seen in their genotype.

APOE ϵ 4 load was the number of ϵ 4 alleles seen in a subject's genotype.

IQR, inter-quartile range; MMSE, Mini-Mental State Examination; CDR, Clinical Dementia Rating.

Overall *p*-value: Result of the Kendall tau test for dependence between SUVR and the demographics variable.

High/low *p*-value: Kruskal Wallis Chi-Squared was used to test between high and low groups for continuous data.

High/low *p*-value: Fishers exact was used to test between high and low groups for categorical data.

Table 3
Candidate proteins of the same UniProt ID

UniProt ID	Proteins	Minimum-Maximum Correlation
P01024	C3; C3adesArg; C3b; iC3b; C3a; C3d	0.468 – 0.964
P02671	Fibrinogen; D-dimer	0.651
P02649	<i>APOE</i> ; <i>APOE3</i> ; <i>APOE4</i>	0.787 – 0.894

machine. Table 3 gives details of the proteins that were collapsed into their first principal component, as detailed previously. In all cases, PC1 accounted for at least 75% of the total variation. The only probe leading to correlations of less than 0.75 for UniProt ID P01024 was that of C3d.

Discovery analysis

Discovery analysis was applied across the entire SOMAscan platform in an attempt to find predictors of dichotomized as well as continuous NAB. Variable selection techniques that included linear and logistic regression and SVMs yielded no predictive models with efficacy of estimating NAB above that of a model built on age and *APOE* ϵ 4 carriage alone. The results of linear and logistic regression methods to predict continuous and dichotomized NAB are given in Supplementary Table 2.

Single protein analysis

Residual plots indicated a reasonable model fit in all cases. We outline below single proteins that pass multiple testing with a *q*-value of less than 0.1.

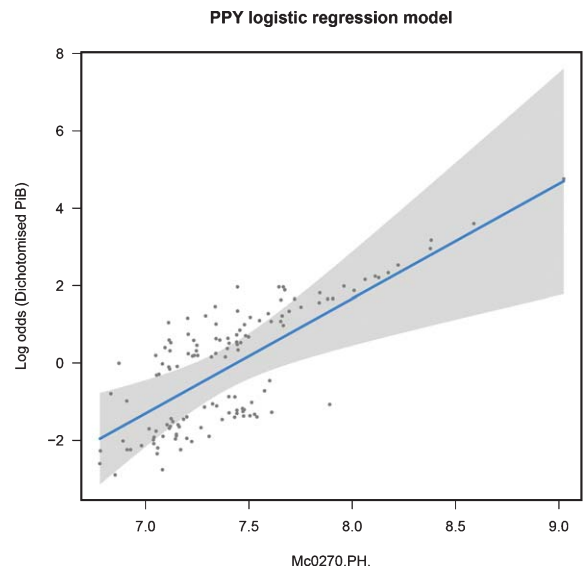


Fig. 2. Regression fit for PPY modeling dichotomized NAB in all samples: training data.

Non-AIBL candidates

When modeling dichotomized NAB, logistic regression analysis showed that PPY was significantly associated with PiB positivity across all samples, passing multiple testing corrections ($\beta = 2.959$, $p = 0.001$, $q = 0.013$). Figure 2 shows the fit of this model in the training data. See Supplementary Table 3 for full results.

AIBL candidates

In control samples, IgM was significant in a linear model with a *q*-value of 0.044 ($\beta = -0.282$, $p = 0.002$). Mirroring the non-AIBL candidate results, PPY

Table 4

Population	Candidates	Method	Test data					
			Accuracy	Accuracy Empirical <i>p</i> -value	Sensitivity	Sensitivity Empirical <i>p</i> -value	Specificity	Specificity Empirical <i>p</i> -value
All	Non-AIBL	Logistic regression [Age, <i>APOE</i> ε4 number, IL13, PPY, APOE PC1, C3 PC1]	0.522	0.833	0.613	0.941	0.444	0.352
		SVM						
	AIBL	Logistic regression [Age, <i>APOE</i> ε4 number, BLC, Clusterin, PPY, C4, Hemopexin]	0.54	–	0.5	–	0.58	–
		SVM	0.612	0.119	0.645	0.78	0.583	0.026
	Age and APOE	Logistic regression [Age and <i>APOE</i> ε4 number]	0.63	–	0.59	–	0.67	–
		SVM	0.567	–	0.774	–	0.389	–
Controls	Non-AIBL	Logistic regression [Age, <i>APOE</i> ε4 number, vWF, PPY, APOE PC1, C3 PC1, Fibrinogen PC1]	0.61	–	0.57	–	0.66	–
		SVM						
	AIBL	Logistic regression [Age, IgM, BLC, Alpha-2-Macroglobulin, PPY, Gelsolin, Factor B, Hemopexin]	0.575	0.693	0.5	0.053	0.636	0.963
		SVM						
	Age and APOE	Logistic regression [Age and <i>APOE</i> ε4 number]	0.65	–	0.7	–	0.63	–
		SVM	0.675	0.083	0.444	0.217	0.864	0.155
	Non-AIBL	Logistic regression [Age, <i>APOE</i> ε4 number, BLC, Clusterin, PPY, C4, Hemopexin]	0.7	–	1	–	0.65	–
		SVM	0.65	–	0.333	–	0.909	–
	AIBL	Logistic regression [Age and <i>APOE</i> ε4 number]	0.55	–	NA**	–	0.55	–
		SVM						
	Age and APOE	Logistic regression [Age and <i>APOE</i> ε4 number]						
		SVM						

PC1, First Principal Component;
SVM, Support Vector Machine.

****All samples are predicted to have low NAB.**

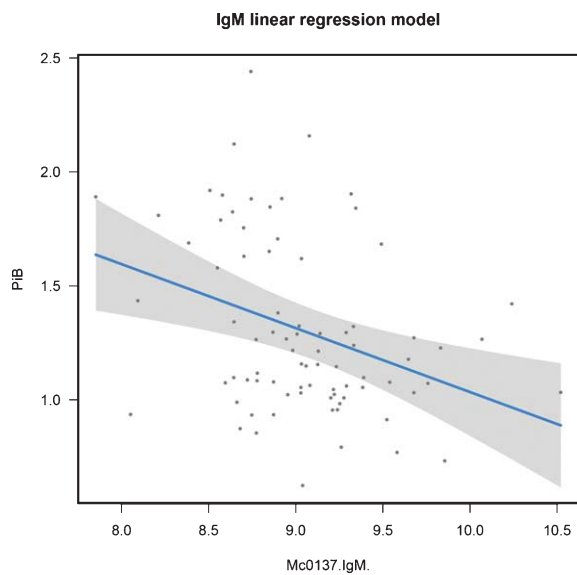


Fig. 3. Regression fit for IgM modeling continuous NAB in control samples: training data.

was significant in a logistic regression model when considering the dichotomized endpoint in all samples ($q = 0.018$). Figures 2 and 3 show the fit of these mod-

els in the training data. See Supplementary Table 4 for full results.

The analysis of continuous NAB against IgM in control subjects was repeated including a flag for presence of diabetes as a covariate ($n = 72$). IgM had an estimate similar to that previously stated ($\beta = -0.232$) and showed a nominally significant p -value ($p = 0.027$). The presence of diabetes flag was not significant ($\beta = 0.127$, $p = 0.586$).

Multiple protein analysis

Continuous NAB

In linear regression analysis across all subjects models of both non-AIBL candidates and AIBL candidates showed improved RMSE (0.490 and 0.497) and R^2 (0.245 and 0.226) values over a model of age and APOE alone (RMSE = 0.500 and $R^2 = 0.219$). The model of non-AIBL candidates achieved significant empirical p -values for RMSE and R^2 through permutation testing with p -values of 0.041 and 0.043, respectively. Model statistics achieved in parametric analysis outperformed those from SVM modeling.

Table 5
Multiple protein analysis: continuous NAB

Population	Candidates	Method	Test data			
			R^2	R^2 Empirical p -value	RMSE	RMSE Empirical p -value
All	Non-AIBL	Linear regression [Age, APOE $\epsilon 4$ number, Albumin PPY]	0.245	0.043	0.49	0.041
		SVM	0.15	–	0.52	–
	AIBL	Linear regression [Age, APOE $\epsilon 4$ number, BLC, C4]	0.226	0.061	0.497	0.058
		SVM	0.15	–	0.53	–
	Age and APOE	Linear regression [Age and APOE $\epsilon 4$ number]	0.219	–	0.5	–
		SVM	0.1	–	0.53	–
Controls	Non-AIBL	Linear regression [Age, APOE $\epsilon 4$ number, vWF, PPY, Plasminogen]	0.241	0.215	0.356	0.226
		SVM	0.23	–	0.36	–
	AIBL	Linear regression [Age, APOE $\epsilon 4$ number, IgM, BLC, Alpha-2-Macroglobulin, HRG, Factor B, Hemopexin]	0.17	0.371	0.392	0.537
		SVM	0.25	–	0.36	–
	Age and APOE	Linear regression [Age and APOE $\epsilon 4$ number]	0.273	–	0.349	–
		SVM	0.02	–	0.41	–

PC1, First Principal Component;
SVM, Support Vector Machine.

Protein models in control samples created through regression analysis did not outperform a model of age and APOE alone. However, all SVM models showed lower RMSE and higher R^2 values than the demographic only model and in most cases also outperformed the parametric methods. For further details of the multiple protein analysis of continuous NAB, see Table 5.

Dichotomized NAB

In parametric analysis across all subjects, the model of AIBL candidates gave a higher accuracy (0.612) than a model of age and APOE alone (0.567). This was driven by an increase in specificity; 0.583 compared with 0.389 in the age and APOE only model. Specificity gave a significant permutation p -value of 0.026.

The model of non-AIBL candidates also showed increased specificity at 0.444 compared to 0.389 in the age and APOE only model. SVM analysis gave increased specificity and decreased sensitivity over parametric analysis in all three models leading to a marginal increase in overall accuracy.

In control samples, logistic regression analysis of AIBL candidates gave improved accuracy and sensitivity compared with the model of age and APOE alone (0.675 and 0.444, respectively, compared with 0.650 and 0.333). The model in non-AIBL candidates also showed increased sensitivity at 0.500. Non-parametric analysis showed improved accuracy over regression analysis in the protein based models driven by an increase in sensitivity. For further details of the multiple protein analysis of dichotomized NAB, see Table 4.

DISCUSSION

In this study, we investigated blood proteins shown in the literature to be associated with A β burden in the brain, using serum samples from AIBL. No proteins were found to pass multiple testing corrections in a discovery analysis so we implemented a candidate based approach.

Two candidate proteins (PPY and IgM) showed association with NAB in the single protein analysis of the AIBL serum SOMAscan dataset. Both had already been identified as significant in the AIBL plasma RBM MAP dataset by Burnham et al. [17].

PPY passed multiple testing corrections for single protein analysis in all samples for the dichotomized endpoint. This provides further evidence for claims made by Chiam et al. who found PPY to be the

most replicated blood protein marker of AD [34]. The direction of association (positive) matched that of Burnham et al. [17], providing some evidence for concordance of the SOMAscan platform with RBM MAP for this protein despite technical differences. This is also consistent with Sattlecker et al. who used SOMAscan to show that plasma PPY levels increase with disease progression in subjects from the combined AddNeuroMed, Alzheimer's Research UK/Maudsley BRC Dementia Case Registry research cohorts [18]. However, the estimate was in the opposite direction to that described by Kiddle et al. [16]. This could suggest a complex relationship of PPY with NAB that we may be unable to describe fully with the statistical approaches applied here. Alternatively, the variation in direction could be due to lower statistical power in Kiddle et al. ($n=71$) or cohort differences, perhaps differences between Americans and Australians or disease stage. Indeed, Lunnon et al. have previously shown that disease associated markers, albeit gene expression markers, can frequently change direction of effect depending on disease stage [35]. The subjects used by Kiddle et al. from ADNI were at a much later disease stage (with only three controls) than the subjects used here.

Doecke et al. have previously shown PPY to be associated with AD diagnosis in the AIBL cohort [36]. Similarly, PPY was found to be associated with diagnosis in ADNI and the Texas Alzheimer's Research Consortium [37, 38]. However, there has been discussion around whether this effect could be a pharmacological response to the use of acetylcholinesterase inhibitors. Unfortunately, we were unable to examine this in the AIBL cohort but the possibility of medication confounding should be investigated in further studies. Chiam et al. use data from the AddNeuroMed study to suggest the effect of acetylcholinesterase inhibitor use is not significant on PPY (Chiam et al., unpublished results).

Additionally, IgM was seen to replicate at the single protein level in control samples only. IgM is an antibody produced by B-cells that appears early in the course of an infection. This pathway of immune response has been linked to AD in previous studies [35]. Furthermore, IgM has been associated with diabetes [39]. When a presence of diabetes flag is included in the IgM model it is not significant. Additionally, the p -value of IgM remains nominally significant at 0.027 indicating that it is unlikely that this significant association is a result of disease confounding.

There were 29 candidates that gave non-significant results. These were: C3, APOE, Fibrinogen, Hap-

toglobin, Albumin, α 1-antitrypsin, α 2-macroglobin, APOA1, C4, Chemokine Ligand 13, Clusterin, Factor B, Factor H, Gelsolin, Hemopexin, HRG, IgG, IL13, IL17, IL3, Leptin, MIP1 α , MMP9, Plasminogen, Transferrin, SAP, VCAM1, vWF, and APP.

Some of these proteins showed significant q -values for model statistics (such as R^2 and accuracy based on permutation testing) although they were not significant in the parametric model. This indicates that greater sample size is needed for future studies of this nature in order to reduce the variation of statistics such as R^2 and hence give a more reliable estimate.

Multiple protein models constructed in this study gave mixed success in explaining variation in brain A β burden. Although some provided increased accuracy or R^2 over models of age and APOE ϵ 4 number alone, the differences were minimal and largely no greater than those provided by some single protein models. LMG variable importance scores from linear regression models also emphasized that age and APOE ϵ 4 number were the most important variables. Consequently, given a larger sample size, an analysis stratified by APOE genotype could be used to reveal more complex relationships between APOE and protein markers.

The four studies that contributed candidates for this analysis used blood plasma samples. However, due to sample availability, we performed SOMAscan analysis on blood serum samples. It is possible that this tissue difference is responsible for the non-replication of some of the candidates; however, there are studies suggesting analyte specific consistency across sample type. O'Bryant et al. show that some blood-based markers of AD, including pancreatic polypeptide, are consistent between plasma and serum [38]. Furthermore, our comparison of ranked lists of proteins associated with age between blood serum (AIBL) and plasma (Twins UK) showed a high level of concordance on the SOMAscan platform.

Differences between the proteomic approaches used here (SOMAscan) and in the literature (Myriad RBM, 2DGE, and mass spectrometry) could be driving the disparity in results between these studies. We see non-significant p -values for similarity when comparing ranked lists of proteins associated with age and gender between SOMAscan and RBM MAP. Due to the similarity in signal between the Twins UK plasma samples and AIBL serum samples (detailed above), this difference is likely platform-driven. While SOMAscan measures the availability of a 3D shape and charge epitope, mass spectrometry measures mass to charge ratio. RBM MAP uses antibodies that could be measuring alternative versions or conformations of proteins to

those measured by SOMAscan. It is likely the SOMAscan platform would show the highest correlation with other immunoassays. This is consistent with the observation that we see the most agreement between AIBL plasma RBM MAP candidates and our findings here using serum SOMAscan (PPY and IgM). A study examining the similarities and differences observed when running samples across these platforms would be useful when interpreting results such as these.

The primary analysis used in this study follows a parametric, linear approach following that of three out of the four studies cited. Burnham et al. used a non-linear, non-parametric analysis. We investigated the potential ability to detect interactions and non-linear relationships, and consequently improve model performance, by building SVM models. These non-parametric models found slightly improved results suggesting that non-linearity and interactions could be important in the relationship between blood proteomics and NAB and should be considered in further studies.

Throughout the analysis presented here we included APOE ϵ 4 number (0, 1, 2) as a covariate. We did not include the APOE genotype itself due to small numbers of samples in the ϵ 2 ϵ 4 and ϵ 4 ϵ 4 groups. We investigated whether grouping subjects with an ϵ 2 ϵ 4 genotype with subjects with an ϵ 3 ϵ 4 genotype was appropriate given that the ϵ 2 allele has been found to be protective and consequently subjects of the two genotypes could behave differently. Exploratory analysis of the PPY and IgM results suggest that the general trend between the ϵ 3 ϵ 4 and ϵ 2 ϵ 4 groups is similar and consequently it is reasonable to combine them in our analysis. Supplementary Figures 2 and 3 illustrate this.

It is clear that the sample size used in our analysis limits the power of any conclusions. An additional factor limiting interpretation of these findings is how representative the cohort used here is compared to potential clinical trial recruitment populations, both in terms of diagnostic groups and prevalence of high A β burden. Differences in prevalence between this cohort and the target populations can lead to inflated estimates of positive predictive value, the proportion of test positives that are true positives [40]. This should be addressed in further work through study of larger and more representative cohorts.

A known disadvantage of stepwise regression analysis is a tendency to over fit to the training data. Several of the multiple protein models created failed to outperform age and APOE ϵ 4 number in test data which could be a suggestion that the stepwise models are over fitting to the training data. Alternatively, this may provide evidence that the candidates considered here provide

minimal predictive information on NAB, above that of age and APOE ϵ 4 load, when measured in serum samples using SOMAscan. Studies of larger cohorts will allow this to be investigated further.

It is promising that we show for the first time that IgM predicts NAB in asymptomatic individuals. However, we note that the majority of candidates investigated in this study had been selected from populations with a variety of diagnoses. Thus, the lack of significance of association with NAB for most candidates in asymptomatic subjects was not unexpected. In further work it would be interesting to perform a discovery analysis on control subjects alone and identify proteins that are found to link with A β burden pre-symptomatically. The proteins identified by Thambisetty et al. are from a control population but of small sample size ($n=57$) [15].

We were surprised to detect a signal for fibrinogen from the SOMAscan technology. Currently it is thought that there should be no fibrinogen present in blood serum samples. This may reflect issues of specificity with the fibrinogen SOMAmer or the presence of low concentrations of fibrinogen in serum samples beneath detection thresholds of other assays. We note that this has been reported before in a study of tuberculosis [41]. As SOMAscan is a relatively novel protein quantification technology, more work is needed to qualify how it compares with other platforms and to identify the binding sites of SOMAmers.

There is a clear link between the development of A β plaques in the brain and AD but this relationship is not exclusive. That is to say, it is not always the case that high NAB indicates AD or vice versa; there are examples of elderly subjects with high A β burden and no cognitive impairment [42]. While this could be explained by misdiagnosis and A β starting a slow AD process in motion, the alternative hypothesis would be that A β is not causally related to late onset AD. However, while the focus of AD clinical trials is predominately anti-A β , it is logical to find markers relevant to those trials. Furthermore, A β burden is appearing in new diagnostic criteria for AD [43]. More work is needed to thoroughly understand the role of brain A β burden in AD to validate the use mechanistic action of A β , it would be interesting to study a cohort of subjects with A β positivity of a model of A β burden as a diagnostic biomarker. To investigate the prior to the development of MCI or AD. Unfortunately, the current study did not have a sufficient number of samples ($n=5$) meeting this criterion to perform such analysis. The need for cohorts of this type is clear in order to advance understanding of the clinical relevance of A β .

If the model statistics achieved in this study are shown to be robust across relevant, larger, and independent populations, these models could be beneficial in acting as a cost-effective enrichment filter between the general population and those predicted to have high NAB. Using a relevant simple blood test as a pre-screening tool could reduce the PET and CSF screening failure rate and hence the cost of trials.

CONCLUSIONS

In this study we investigated blood proteins previously found to be associated with A β burden in the brain using serum samples from AIBL and the SOMAscan proteomics technology. Two candidate proteins (PPY and IgM) showed association with NAB in the AIBL serum SOMAlogic dataset. Notably, IgM was found to associate with continuous NAB across control subjects, suggesting it may have utility for predicting A β -positive asymptomatic individuals. There are several reasons for a lack of significance for the other candidates including platform differences and the use of serum rather than plasma samples. To investigate the possibility of technical differences causing lack of further replication, further studies are required.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-150020>.

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